Cell-Based Screen for Identification of Inhibitors of Tubulin Polymerization

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This assay is based on morphological changes of rat glioma cells treated with db-cAMP. The db-cAMP treatment induces a tubulin-dependent change causing the cells to acquire a spherical shape. Pretreatment with tubulin inhibitors brings about the disintegration of tubulin polymer and/or prevents its polymerization. Cells with inhibited tubulin fail to respond to db-cAMP treatment. Cells treated with inhibitors of tubulin polymerization are then separated from the spherical cells by aspiration. A semiautomated scanning procedure evaluates the final culture density and yields graphical data.

Polymerized tubulin dimers exist in equilibrium with the free tubulin dimers. Compounds such as colchicine, vincristine, etc., bind free tubulin dimers and shift the equilibrium in favor of depolymerized tubulin to inhibit microtubule formation.[1] Disruption of microtubule function can interrupt many cellular processes. Disruption of mitotic spindles (composed of tubulin) appears to be particularly important for anticancer activity with treated cells accumulating in the M phase.[2]

The ability of dibutyryl-cAMP (db-cAMP) to induce morphologic change in rodent neural tumor cell lines was recognized early.[3,4] Igarashi and co-workers described this activity in rat glioma cell lines.[5] Many rat glioma lines, like the C6 cell line used here, respond very rapidly to db-cAMP treatment and assume a spherical morphology within 60 min.[5] This response is due to the polymerization of tubulin. When such cells are treated with inhibitors of tubulin polymerization they fail to undergo the db-cAMP-induced morphology change.[5]

A natural products screen based on these observations was established in 1982.[6] It relied on subjective evaluation of db-cAMP-induced morphological changes and provided no objective quantified data. Nevertheless, this assay has proven useful in the identification of at least one additional class of mitotic inhibitor[7] and has been used to identify compounds with cAMP elevating activity.[8] The assay described here further develops this screen by adapting it to microtiter plate technology and thus rendering the results quantifiable by an objective measure. In this assay the spherical cells that have responded to the db-cAMP are differentiated from cells that have not on the basis of their adhesion to the culture substratum. The spherical cells have a relatively small area of attachment and can be aspirated off the culture plate under conditions that will not wash off unresponsive cells (e.g., cells treated with inhibitors of tubulin polymerization). The number of cells remaining after aspiration can be quantified by treatment with MTT. The MTT is metabolized by the remaining cells to a formazan product that absorbs 450 nm light. This absorbance is measured in a 96-well microtiter plate reader, and the data are plotted following conversion in a personal computer.

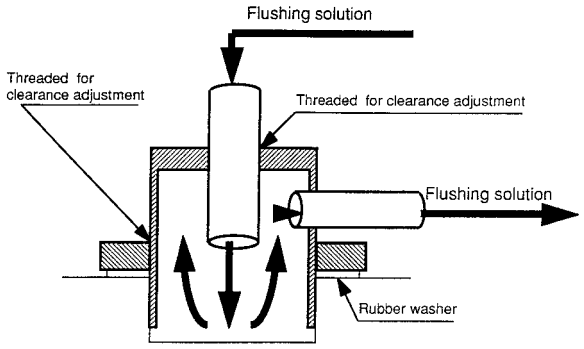

Figure 1. Schematic of aspiration tool.

Results and Discussion

This assay constitutes a useful mechanism-based screen for the evaluation of cytotoxic compounds. It is easy to perform and requires no specialized equipment other than the aspiration tool (Figure 1). By utilizing 96-well microtiter plate technology it can rapidly provide quantified comparisons between unknown compounds and known inhibitors of tubulin polymerization such as colchicine used here. It is anticipated that novel compounds identified by this protocol would have their tubulin activity confirmed in secondary assays that have been developed to assess tubulin active compounds.[9]

C6 cells quickly change their morphology when db-cAMP is added, as previously reported for other glioma and blastoma cells.[10−12] Prior to introduction of db-cAMP, they exhibit glial (fibroblastic) morphology characterized by elongated stoma with two or more processes. After db-cAMP addition, they become spherical with thin neurites. The db-cAMP-induced morphological change takes place quickly. Within minutes, changes are visible under the microscope.[13,14] Cells pretreated

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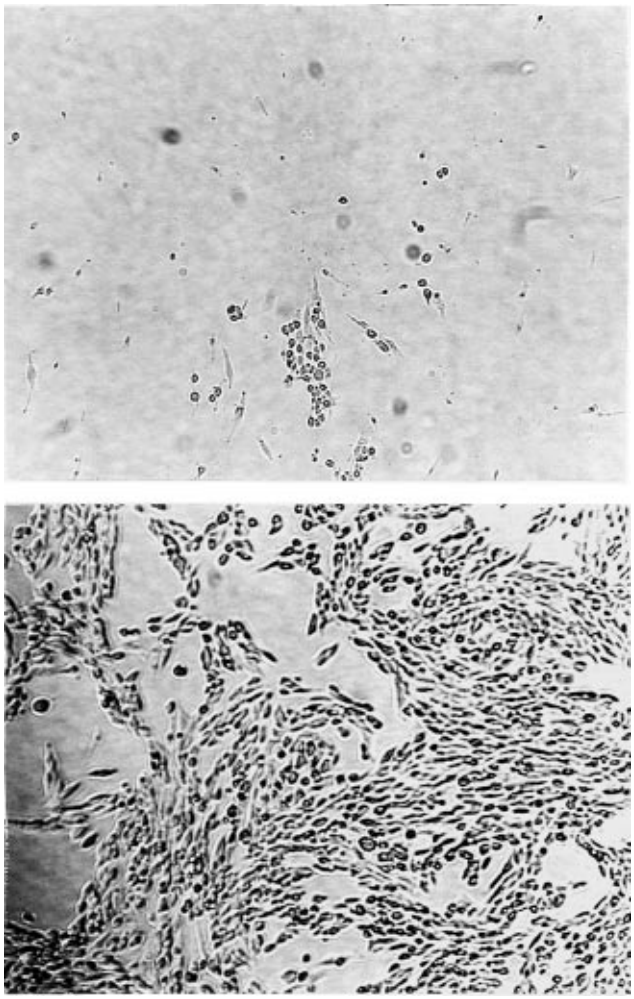

Figure 2. (Top) aspirated culture treated with db-cAMP only (magnified 40×). (Bottom) aspirated culture treated with colchicine and db-cAMP (magnified 40×).

with a tubulin inhibitor do not respond to the db-cAMP treatment. Visual inspection of the plates upon aspiration confirms that cells treated with a tubulin inhibitor prior to db-cAMP treatment are still in place while cells treated with db-cAMP only are washed away (Figure 2, top, bottom).

The validity of this assay was tested by semiblind screening of the following compounds: actinomycin D, ARB-2, BCNU, cisplatin, citochalasin B, colchicine, curacin A, cyclohexamide, daunomycin, doxorubicin, etoposide, latrunculin, melphalan, mitomycin, mitotane, mitoxantrone, nalidixic acid, novobiocin, streptozotocin, taxol, teniposide, vinblastine, and vincristine. Of these, vinblastine, vincristine, colchicine, and curacin A showed tubulin polymerization-inhibiting activity at relatively low concentrations. Etoposide and ARB-2 (a modified mebendazole[15,16]) also indicated tubulin inhibiting activity, but at higher concentrations. None of the other test compounds indicated tubulin-directed activity. Figure 3 shows the results obtained by screening various cytotoxic agents in this assay.

All compounds were also tested in a 3-day fractional survival assay.[17] The 50% lethal concentrations of the tubulin active compounds are shown in Table 1. The tubulin inactive compounds also proved toxic at concentrations tested for tubulin activity (data not shown). This is consistent with the dogma that these tubulin inactive compounds are cytotoxic by other mechanisms.

To address the concern that extremely toxic compounds could induce a false negative result in this assay, the entire set of 23 compounds were retested in the procedure with omission of db-cAMP. If cell killing caused cellular release within the 5 h time frame of this protocol, the jet-aspiration would rinse them away even in the absence of db-cAMP. Thus, even tubulin-active compounds would appear inactive. None of the test compounds, even at 50 μg/mL, caused any cell release in the absence of db-cAMP at 5 h.

Colchicine, vincristine, vinblastine, and curacin A are well-documented tubulin inhibitors. Etoposide is a semisynthetic podophyllotoxin, a class of cytotoxic compounds identified as tubulin inhibitors. Etoposide itself is thought to exert its cytotoxic-anticancer activity via topoisomerase II inhibition.[18,19] These results suggest that at very high concentration (680 μM) etoposide still retains some antitubulin activity. ARB-2 is a new compound. It is a methylated analog of mebendazole and thus a member of a class of known tubulin inhibitors.[20,21] Cisplatin and melphalan also scored positive, but at concentrations greater than 50 μg/mL. These compounds could easily be distinguished from tubulin inhibitors because they did not prevent the db-cAMP-induced morphological change. It is likely that the cross-linking activity of these agents increased the attachment of the spherical cells to the culture substratum.

Taxol and latrunculin scored negatively in this assay (e.g., Figure 3D). Taxol is a known tubulin-active compound that has a promoting effect on tubulin polymerization. Taxol treatment altered C6 cell morphology only slightly. Also, the taxol-treated cells did not appear to respond fully to db-cAMP. Nevertheless, this intermediate cellular morphology was rinsed away during the aspiration step of the procedure and resulted in no signal from taxol-treated cells. Reported differences[13] between the effects of taxol and colchicine on cellular arborization are consistent with this observation. Latrunculin is a cytotoxic marine natural product that is known to disrupt actin filaments.[22,23] It was not active in this assay either.

Experimental Section

A C6 rat glioma culture was purchased from ATCC #107-CCL and maintained in α-MEM medium consisting of 7.5% α-calf fraction (HyClone), 2.5% fetal bovine serum (HyClone), 1.5% penicillin-streptomycin (Sigma Chemical Co.), and 0.2% nystatin suspension (Sigma Chemical Co.). Test compounds were dissolved in dimethyl sulfoxide (DMSO) purchased from Sigma Chemical Co. Morphological changes were achieved with the use of N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (db-cAMP) from Sigma Chemical Co. Cultures were aspirated with phosphate-buffered saline PBS containing 0.20 g/L KCl, 0.20 g/L KH_2PO_4, 8 g/L NaCl, and 2.16 g/L $Na_2HPO_4\times 7\ H_2O$. Cultures were incubated in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in McCoy's medium, which also contained 2.5% fetal bovine serum (HyClone), 7.5% α-calf fraction (HyClone), 1.5% penicillin−streptomycin (Sigma Chemical Co.), and 0.2% nystatin suspension (Sigma Chemical Co.). Ultraviolet absorbance was determined using a Bio-RAD Model 450 microplate reader.

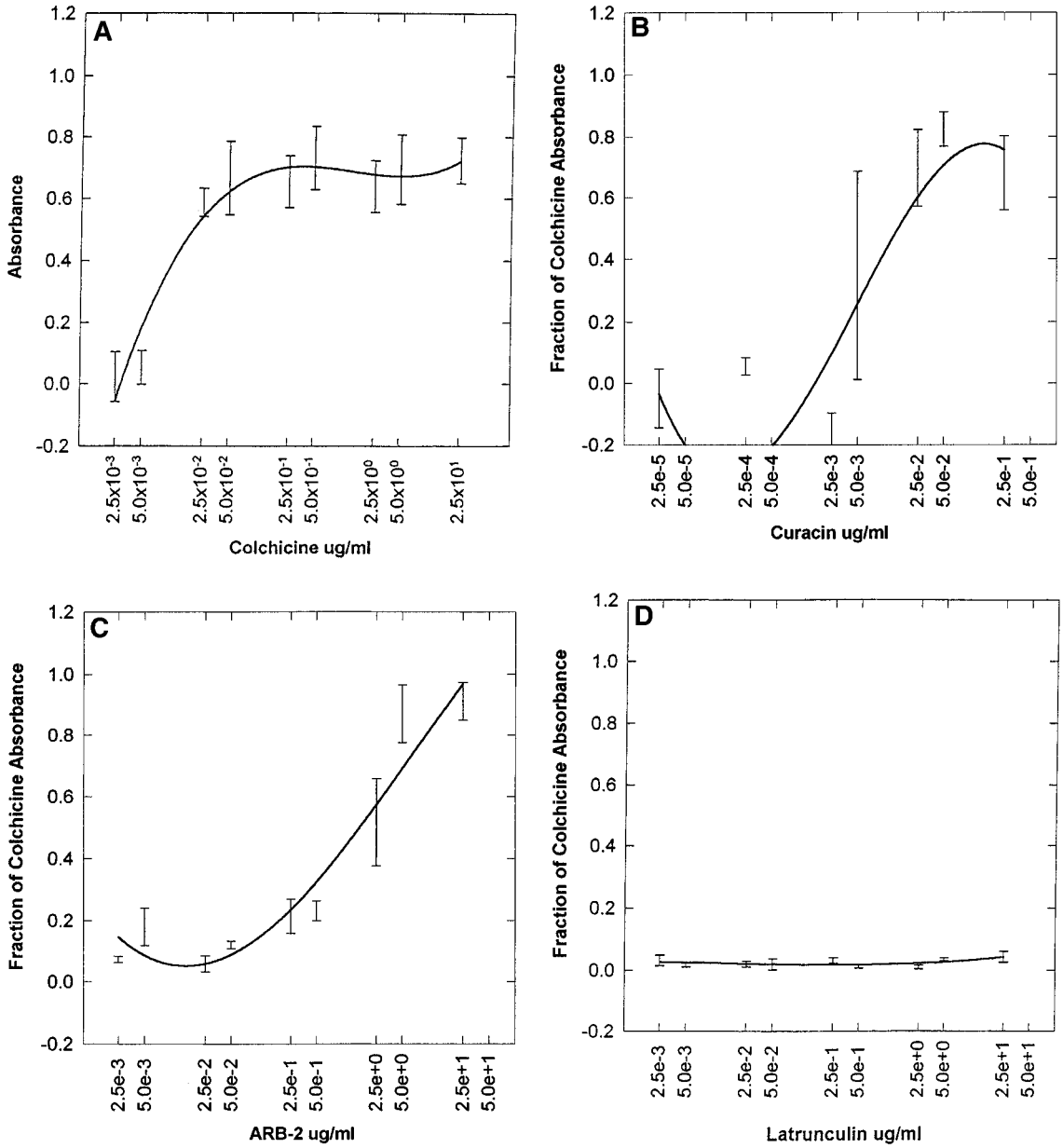

Figure 3. (A) Absorbance of wells treated with colchicine. (B) Absorbance of wells treated with curacin A expressed as a fraction of the absorbance of wells treated with 25 μg/mL of colchicine. (C) Absorbance of wells treated with ARB-2 expressed as a fraction of the absorbance of wells treated with 25 μg/mL of colchicine. (D) Absorbance of wells treated with latrunculin expressed as a fraction of the absorbance of wells treated with 25 μg/mL of colchicine.

Table 1. Effective and Lethal Concentrations of Compounds Active in This Assay[a]

	EC_{50} at 4 h (M)	LC_{50} at 3 days
vinblastine	8.2×10^{-6}	6.6×10^{-8}
vincristine	6.2×10^{-6}	3.2×10^{-7}
colchicine	1.0×10^{-7}	2.0×10^{-7}
curacin	5.1×10^{-7}	1.1×10^{-8}
etoposide	6.8×10^{-4}	1.2×10^{-7}
ARB-2	3.5×10^{-4}	2.7×10^{-5}
taxol	no conformational effect	2.3×10^{-8}

[a] EC, effective concentration in the tubulin assay; LC, lethal concentration at 3 days.

General Experimental Procedures. C6 rat glioma cells were transferred into a 96-well plate at 5×10^4 cells in 200 μL of α-MEM medium per well and allowed to incubate for 24 h. Test compound was then added to all but one column of wells. Colchicine at 25 μg/mL final concentration was added to this column as a positive control. The toxin dilutions ranged from 2.5×10^{-3} to 50 μg/mL final concentration (sometimes lower concentrations were used for exceptionally active compounds). Four hours later 1 μL of 0.5 M db-cAMP was added to all wells. All dilutions were tested in quadruplicate in each experiment. Experiments were repeated three to four times.

After 45 min incubation the db-cAMP-induced morphological change was complete, and all wells were aspirated. A customized tool (Figure 1) was used to deliver a flow of PBS buffer into each well as it was aspirated. The dynamic effect of the buffer flow combined with the aspirating vacuum had to be relatively even over the bottom of the microtiter plate culture well for optimal results. Upon aspirating with 1.7×10^4 Pa vacuum for 5 s, each well was supplied with 100 μL of McCoy's medium and 11 μL of 5 mg/mL stock MTT in PBS solution. The glioma cells metabolize MTT to a dark formazan dye.[24,25] Four hours were allowed for the metabolite to accumulate. Using a multichannel

pipette, 100 μL of solution containing 0.04 N HCl in 2-propanol was added to each well. The plate was placed into a BioRad model 450 reader and scanned for light absorbance at 450 nm. The data were analyzed graphically by computer.

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